Plasticizers used in food-contact materials affect adipogenesis in 3T3-L1 cells

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Abstract

Recent studies suggest that exposure to some plasticizers, such as Bisphenol A (BPA), play a role in endocrine/metabolic dispruption and can affect lipid accumulation in adipocytes. Here, we investigated the adipogenic activity and nuclear receptor interactions of four plasticizers approved for the manufacturing of food-contact materials (FCMs) and currently considered safer alternatives. Differentiating 3T3-L1 mouse preadipocytes were exposed to scalar concentrations (0.01-25 μM) of DiNP (Di-iso-nonyl-phthalate), DiDP (Di-iso-decyl-phthalate), DEGDB (Diethylene glycol dibenzoate), or TMCP (Tri-m-cresyl phosphate). Rosiglitazone, a well-known pro-adipogenic peroxisome proliferator activated receptor gamma (PPARγ) agonist, and the plasticizer BPA were included as reference compounds. All concentrations of plasticizers were able to enhance lipid accumulation, with TMCP being the most effective one. Accordingly, when comparing *in silico* the ligand binding efficiencies to the nuclear receptors PPARγ and retinoid-X-receptor-alpha (RXRα), TMPC displayed the highest affinity to both receptors. Differently from BPA, the four plasticizers were most effective in enhancing lipid accumulation when added in the mid-late phase of differentiation, thus suggesting the involvement of different intracellular signalling pathways. In line with this, TMCP, DiDP, DiNP and DEGDB were able to activate PPARγ in transient transfection assays, while previous studies demonstrated that BPA acts mainly through other nuclear receptors. qRT-PCR studies showed that all plasticizers were able to increase the expression of CCAAT/enhancer binding protein β (*Cebpβ)* in the early steps of adipogenesis, and the adipogenesis master gene *Pparγ2* in the middle phase, with very similar efficacy to that of Rosiglitazone. In addition, TMCP was able to modulate the expression of both Fatty Acid Binding Protein 4/Adipocyte Protein 2 (*Fabp4/Ap2)* and Lipoprotein Lipase (*Lpl)*

transcripts in the late phase of adipogenesis. DEGDB increased the expression of *Lpl* only, while the phthalate DiDP did not change the expression of either late-phase marker genes *Fabp4* and *Lpl.* Taken together, our results suggest that exposure to low, environmentally relevant doses of the plasticizers DiNP, DiDP DEGDB and TMCP increase lipid accumulation in 3T3-L1 adipocytes, an effect likely mediated through activation of PPARγ and interference at different levels with the transcriptional cascade driving adipogenesis.

Keywords: plasticizer; endocrine disruptor; phthalates; adipogenesis; nuclear receptors; lipid accumulation

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1. Introduction

Obesity is the fastest growing health problem in Europe and worldwide. In the European Union, overweight affects between 36% and 67.5% of adults, while obesity affect between 10% and 28% of adults (last update 2014) [1]. In addition to genetic factors, life style factors such as excessive caloric intake, high fat diets, and low physical activity contribute to obesity. However, there is also increasing evidence that environmental pollutants including endocrine-disrupting chemicals (EDCs) may contribute to the development of obesity and metabolic disorders. A subset of EDCs have been named ”obesogens” or “metabolic disruptors” [2–5], because of their ability to promote adiposity by altering fat cell development and increasing energy storage of fat tissue, and because of their implication in metabolic syndrome and obesity [6].

The EU regulation (1907/2006 and subsequent updates) regarding the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) has identified so far 181 substances of very high concern (SVHC) for the environment and human health (last update January 2018). Several SVHC are plasticizers, a class of diverse additives used in plastics production, that are poorly bound or not bound to the polymers. These features facilitate their migration from food-contact materials (FCMs) and several household plastic items, thus coming in contact with humans through food consumption, skin absorption and inhalation [7]. FCMs, including plastic packaging, are not generally perceived to be a chemical health threat when compared to pesticides, veterinary drugs or heavy metals arising from agricultural practices or environmental contamination. However, within the last decade it has been increasingly reported that certain FCMs can act like EDCs [8]; a good example are plastic additives used in food containers like Bisphenol A (BPA), a substance recently

included in the SVHC list and whose impact on the endocrine system has been increasingly reported [2,3].

The EFSA (European Food Safety Authority) regulation 10/2011 has provided a list of plasticizers permitted in EU for FCMs manufacturing, which has become a useful source of alternatives to currently used SVHC. In the present work, we focused our attention on four plasticizers employed in food packaging: Di-iso-nonyl-phtalate (DiNP), Di-iso-decyl-phtalate (DiDP), Diethylen glycol dibenzoate (DEGDB), and Tri-m-cresyl phosphate (TMCP). Notably, DiNP and DiDP are comprised in the EFSA list of permitted compounds and are indeed among the most used in the plastic market (33% United States; 63% European Union) as substitutes of di(2-ethylhexyl) phthalate (DEHP), a substance classified as SVHC [9,10]. DEGDB is another emerging plasticizer designed to substitute phthalates, since it is considered more eco-friendly due to its biodegradation pathways [11]. Tri-cresyl phosphates, such as tri-m-cresyl phosphate (TMCP), are mainly used as substitutes of the plasticizers polybrominated diphenyl ethers (e.g. BDE-47) [12]. Along with the increased usage of these SVHC substitutes as alternative plasticizers, new biomonitoring data are becoming available associating the exposure to these chemicals with adverse effects in living beings. Notably, DiNP and DiDP have both been associated with increased insulin resistance in adolescent cohorts [13] and in general with several different adverse effects after peri- and post-natal exposure [14]. Interestingly, *in silico* approaches demonstrated that DiNP and DiDP can act as ligands of human peroxisome proliferator activated receptor γ (PPARγ) and retinoid-X-receptor-α (RXRα), possibly triggering a cascade of intracellular events [15]. DiDP is also a confirmed modulator of PPAR:RXR-dependent gene expression pathways in fish hepatocytes [16]. Similarly, TMCP was found to affect lipid/cholesterol

metabolism through a functional interplay between PPARs and liver X receptor (LXR) in a fish *in vitro* system [17]. Also, in fish DEGDB was demonstrated to have high affinities for PPARα, RXRα and LXR, showing the ability to modulate PPARα transcriptional pathways [18].

The 3T3-L1 preadipocyte cell line has proved to be a useful tool to study *in vitro* mechanisms by which obesogens can affect lipid accumulation and adipocyte differentiation. In 3T3-L1 cells, these two processes are regulated by a strict transcriptional activity in which PPARγ is the master regulator [19]. During adipocyte differentiation, three different time windows can be distinguished, each one characterized by the upregulation/activation of a different set of transcription factors: an early phase of induction, characterized by the upregulation of *Cebp* (CCAAT/enhancer binding protein) β and δ and the activation of *Cebp*β and *Rxr*s; a middle phase, with RXRα and PPARγ2 as obligate heterodimers; a late phase, where adipocyte specific genes such as *Fabp4/Ap2* (Fatty Acid Binding Protein 4/Adipocyte Protein 2), *Lpl* (Lipoprotein Lipase), *AdipoQ* (adiponectin) and leptin are upregulated [20–22]. Several studies have shown how environmental chemicals can perturb this intracellular cascade by targeting transcription factors and consequently enhance or decrease adipogenesis [5,6,22–24]. For example, certain EDCs may target PPARγ by binding to it directly to activate downstream cascades leading to enhanced lipid accumulation or by increasing PPARγ expression to favour its activation [24].

In the present work we used 3T3-L1 preadipocytes to investigate the possible adipogenic effects of plasticizers considered safe SVHC substitutes and used in FCMs manufacturing. First, we evaluated possible modifications in lipid accumulation following exposure to scalar concentrations of the plasticizers DiNP, DiDP, DEGDB and TMCP. Since adipogenesis

occurs in 3T3-L1 with a defined timeline of transcription factors and receptors activity, we also evaluated the possible different effects of plasticizer exposure alternatively during 3T3-L1 early or mid-late differentiation. We then verified, by *in silico* molecular docking analysis and reporter gene assays, the ability of these molecules to bind and activate the major transcription factor involved in adipogenesis, namely PPARγ. To better understand the intracellular mechanisms underlying the changes in the adipogenic process, we investigated the regulation of the expression of genes belonging to the early, mid and late phase of adipocyte differentiation.

2. Material and Methods

2.1. Chemicals/Reagents

All the reagents for cell culture (including medium supplements), Oil Red O (CAS Number 1320-06-5), Rosiglitazone (BRL49653; CAS Number 122320-73-4, purity ≥98%), DiNP (di-iso-nonyl-phtalate; CAS Number 28553-12-0, purity ≥99%), DiDP (di-iso-decyl-phtalate; CAS Number 26761-40-0, purity ≥99%), DEGDB (diethylene glycol dibenzoate; CAS Number 120-55-8, purity 90%), TMCP (tri-m-cresyl phosphate; CAS Number 563-04-2) and BPA (Bisphenol A; CAS Number 80-05-7, purity ≥99%) were obtained from Sigma Aldrich (USA).

2.2. 3T3-L1 culture and adipocyte differentiation experiments

3T3-L1 preadipocytes (ATCC® CL-173™; ATCC, USA) were cultured in Dulbecco's modified

Eagle's medium high-glucose (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. $2x10^4$ cells/well were seeded in 24-well plates. Two days after reaching confluence (day 0), cells were exposed to the differentiation medium (MDI; DMEM containing 10% fetal bovine serum, 1 µg/mL insulin, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine). Two days later (day 2), MDI medium was replaced with maintenance medium (MM; DMEM 10% FBS, 1 µg/mL insulin). Fresh medium was provided every two days. Experiments were ended after 10 days from the beginning of the differentiation (day 10).

Cells were exposed to the following plasticizers: DiNP, DiDP, DEGDB, TMCP or BPA at concentrations ranging from 0.01 to 25 µM, that were excluded to be toxic by visual analysis. 100 nM Rosiglitazone was used as a positive control. All the chemicals were dissolved in 100% DMSO as vehicle, and cells were exposed to a final concentration of 0.1% DMSO. Cells were treated with chemicals alternatively from day 0 to day 10 (whole differentiation period treatment), from day 0 to day 2 (early phase treatment), or from day 2 to day 10 (middle-late phase treatment). Control cells were kept in MDI plus 0.1% DMSO from day 0 to day 2 and in MM plus 0.1% DMSO from day 2 to day 10.

Three independent replicates were set in each experiment; experiments were repeated three times at different passage numbers (p8-p11).

2.3. Quantification of adipocyte lipid accumulation

Lipid accumulation in 3T3-L1 adipocytes was determined by quantitative Oil Red O (ORO) staining at day 10. Oil Red O was dissolved in isopropanol overnight at a concentration of 0.35%, followed by 0.2 µm filtration, dilution in water to a final concentration of 0.2%, and

refiltration. Adipocytes were washed twice with PBS, then they were fixed in 10% paraformaldehyde for 10 min at room temperature. Cells were washed with ddH_2O, allowed to dry, and stained with ORO solution for 20 min. Following several washes with ddH_2O, plates were dried at room temperature; ORO was then eluted in 100% isopropanol, and absorbance at 500 nm was measured using a microplate reader (BioRad, USA). The mean of 8 absorbance readings (technical replicates) was calculated for each sample; three independent plate replicates were set in each experiment and experiments were repeated three times. Variations in lipid accumulation were expressed as fold changes of the absorbance of treated cells relative to the absorbance of control cells; controls were assigned a value of 1.

Results are expressed as the mean of the values obtained in the three independent experiments ± standard error of the mean (SEM).

2.4. Molecular docking studies

Molecular docking analysis were performed using Autodock Vina 1.1.2 [25] on an Intel Core i7/Mac OS X 10.9 – based platform, setting a docking zone of 24, 26, and 28 points (in the x, y, and z directions) and of 26, 25, and 27 points with a grid spacing of 1 Å over the human PPARγ and RXRα binding site, respectively.

The crystallographic structures of PPARγ and RXRα receptors were obtained from the Protein Data Bank [26]: PPARγ 1I7I.pdb [27], RXRα 3DZY.pdb [28]. The molecular structures of ligands were obtained from the PubChem database [29] and minimized (with a universal force field, UFF, and a conjugate gradient algorithm until a ΔE lower than 0.001kJ/mol) using the Avogadro software (Version 1.1.0;

http://avogadro.openmolecules.net/) [30].

The affinity constants, expressed as equilibrium dissociation constants (K_d), were determined analysing the 10 best complexes, obtained for each ligand from Autodock Vina, with the NNScore algorithm, version 2.0 [31].

All models and images were rendered using UCSF Chimera software, version 1.11 [32], whereas 2D ligand interaction diagrams were obtained using Maestro software, version 10.6 (Schrödinger, LLC, USA).

2.5. Transfection and reporter gene assays

HepG2 human hepatoblastoma cell line (ATCC® HB-8065™; ATCC, USA) was used for gene reporter assays; cells were plated on a 24 well plate and then transfected with the following constructs [33,34]: (1) 1.5 µg DR1-Luc (containing a direct repeat 1 upstream of luciferase gene), (2) 100 ng pCMV-βgal (pCMV-β-galactosidase normalization plasmid), and (3) 400 ng pcDNA3-PPARγ (an expression vector for human PPARγ) using Lipofectin (Invitrogen). As described previously [35], cells were treated with the indicated ligands 24 hrs post transfection and assayed for luciferase activity 24 hrs post-treatment. Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

2.6. Gene expression analysis

Cells were exposed from day 0 to 25 µM DiDP, DEGDB, TMCP or 100 nM Rosiglitazone; control cells were treated with 0.1% DMSO. Three independent replicates were set in each experiment; experiments were repeated three times. Total RNA was isolated from control and treated 3T3-L1 cells at day 2, day 4 and day 8. Briefly, cells were washed with PBS and

Tri-Reagent (Sigma, USA) was used for RNA extraction following manufacturer guidelines. qReal-Time PCR was performed using Superscript III Platinum One-step qRT-PCR system (Invitrogen, USA) and the thermal cycler Rotor Gene Q (Qiagen, Germany). Intron-spanning primers for representative genes were designed with Primer-BLAST software (NCBI, USA) and are listed in Table 1. Each sample was analysed in three technical replicates containing 50 ng of total RNA. The relative quantification of gene expression was done using a standard curve that was built by pooling all the RNA samples and making serial dilutions (range: 200-6.25 ng of total RNA). The amplicon concentrations were expressed in arbitrary units and were normalized for the expression of *β-actin*, a commonly used housekeeping gene, proved to be a suitable reference gene for qRT-PCR expression studies in 3T3-L1 cells [36]. For each gene, the mRNA expression of the samples was reported as fold changes relative to the expression of control cells; controls were assigned a value of 1.

2.7. Statistical analysis

Statistical analysis was performed with SPSS software (version 24; IBM, USA). All data were analysed with one-way ANOVA plus Tukey or Bonferroni post-hoc test ($p<0.05$). Data were expressed as fold changes versus control ± standard error of the mean (SEM) or ± standard deviation (SD); controls were assigned a value of 1.

3. Results

3.1. The plasticizers DiNP, DiDP, DEGDB and TMCP enhance lipid accumulation in 3T3-L1 cells

We evaluated the effect of four plasticizers belonging to different chemical categories (the phthalates DiNP and DiDP, the benzoate ester DEGDB and the organophosphate TMCP) on adipocyte differentiation by assessing lipid accumulation using Oil Red O (ORO) staining. The plasticizer BPA (Bisphenol A), whose well-documented pro-adipogenic effects have been ascribed to multiple pathways [37], was included as a reference compound; another reference molecule included in the study was Rosiglitazone (BRL49653), because of its well-defined agonist activity toward PPARγ [24]. 3T3-L1 preadipocytes were induced to start adipogenic differentiation and were treated throughout differentiation with vehicle only (0.1% DMSO) or with scalar concentrations (0.01-25 μM) of each plasticizer, while Rosiglitazone was used at a concentration of 100 nM, selected according to published data [6,24,38]. At the end of the experiment (day 10), lipid accumulation was measured by ORO lipid staining and quantification (Fig. 1). As expected, 100 nM Rosiglitazone-exposed cells displayed a strong enhancement (about 7 folds) in lipid accumulation in respect to untreated cells (cultured in MDI-MM medium containing 0.1% DMSO). BPA exerted a clear dose-dependent enhancement of lipid accumulation, the highest concentration (25 μM) being markedly more effective in inducing lipidogenesis than lower concentrations (5 folds for 25 μM versus 1.2-1.8 folds for 0.01-10 μM). Interestingly, also DiNP, DiDP, DEGDB, and TMCP led to a significant increase in lipid accumulation at all tested concentrations. Although lower than the maximal effect reached by the highest doses of BPA, the increase induced by plasticizers was about 20-50% compared to control cells, with TMCP being the most effective plasticizer at all concentrations.

3.2. Plasticizers are more effective in enhancing lipid accumulation when

administered during mid-late differentiation

Since lipidogenesis occurs in 3T3-L1 cells with a defined timeline of transcription factors and receptors activity, we tried to identify windows of susceptibility to plasticizer exposure. For this purpose, plasticizers were added at the lowest concentration tested (0.01 µM) alternatively during the early (day 0-2) or the mid-late differentiation (day 2-10) and lipid accumulation was measured by ORO staining at day 10. An increase in lipid accumulation was observed both when 3T3-L1 cells were treated with plasticizers during the early or the mid-late differentiation (Fig. 2). However, the highest effect on lipidogenesis was reached when plasticizer administration was performed during the mid-late differentiation, except for BPA, for which no statistically significant differences were seen between the two phases. Notably, when administered at 0.01 µM during the mid-late differentiation, BPA resulted the least effective molecule in inducing lipidogenesis, while TMCP was the most effective one. As a matter of fact, exposure to TMCP at days 2-10 was 37% more effective than exposure at days 0-2 (2.33 versus 1.70 folds relative to control), indicating that the mid-late differentiation is considerably more sensitive to TMCP.

3.3. Computational analysis predicts specific interactions of the plasticizers with PPARγ and RXRα

Metabolic disruptors are known to control lipidogenesis and adipocyte differentiation interacting with transcription regulators of gene networks, the main of which belong to the PPAR and RXR receptor families. Since our above-reported results show that plasticizers can enhance *in vitro* 3T3-L1 preadipocytes lipid accumulation, we evaluated if these plasticizers could potentially act via an interaction with the nuclear receptors PPARγ and

RXRα. *In silico* molecular docking analysis, that consider the affinity and the geometry of binding, actually showed the capability of DiNP, DiDP and TMCP to specifically bind the PPARγ receptor with affinities ranging in the submicromolar order; as expected, BPA showed a lower affinity for PPARγ, in respect to the other plasticizers. All the ligands analysed showed a higher binding affinity with RXRα, although their predicted equilibrium dissociation constants for PPARγ are in the same order of magnitude (Table 2). To validate the molecular docking procedure, we added Rosiglitazone to the ligands set and found a predicted equilibrium dissociation constant for PPARγ highly comparable to the K_d value already published [39]. Moreover, the molecular docking model of the best predicted Rosiglitazone/PPARγ complex and the crystallographic structure of this complex (4EMA.pdb) [40] are extremely comparable (data not shown), on the basis of both orientation and average distance of each atom of the ligand (RMSD value = 1.05Å). Among the molecules analysed, TMCP resulted to be the best ligand for PPARγ and RXRα receptors, showing two equilibrium dissociation constants comparable to those of Rosiglitazone. Molecular docking analysis between TMCP and the two receptors ligand binding domains showed that TMCP is exclusively stabilized by non-polar interactions and, in particular, it could establish a pi-pi stacking interaction with Arg^{288} of PPARγ and with Phe^{313} of RXRα (Fig. 3).

3.4. Plasticizers can transactivate PPARγ

We confirmed the ability of the plasticizers TMCP, DiDP, DiNP and DEGDB to bind and activate PPARγ by examining their capacity to induce PPARγ-driven reporter expression following transient transfection of HepG2 cells with pcDNA3-PPARγ. In this assay, all

plasticizers significantly induced PPARγ-driven reporter activity at a concentration of 25 µM, with DiNP and TMPC being already active at 10 µM (Fig. 4). The maximal activity was reached by 25 µM TMCP, that lead to an induction of 2.5 folds, corresponding to about half of the induction obtained by 10 µM Rosiglitazone.

3.5. Plasticizers modulate the expression of adipogenic marker genes

Differentiation of 3T3-L1 preadipocytes, similarly to what occurs *in vivo*, involves a transcriptional cascade initially activated by an adipogenic cocktail (MDI, see Methods) inducing, among others, the transcription factor *Cebpβ (early phase* of differentiation*).* CEPBβ is a direct activator of *Pparγ* transcription *(mid phase),* and 7-12)PPARγ in turn binds as an obligate heterodimer with the nuclear receptor RXR to numerous promoter sites of adipocyte specific genes (*late phase*), including *Fabp4/Ap2* and *Lpl*. In the effort to further elucidate the mechanisms of plasticizer action on preadipocyte differentiation, we analysed by qReal-Time PCR the expression of *Cebpβ*, *Rxrα, Pparγ2, Fabp4/Ap2* and *Lpl* transcripts at day 2, day 4 or day 8 post-induction. 3T3-L1 cells were exposed to 100 nM Rosiglitazone or to 25 µM TMCP, DiDP and DEGDB, a concentration able to induce the highest lipid accumulation in the absence of cytotoxic effects.

At day 2 (Fig. 5, upper panel), corresponding to the early phase of adipogenic differentiation, all the tested molecules were able to enhance the expression of the *Cebpβ* transcript, suggesting that Rosiglitazone, TMCP, DiDP and DEGDB can influence the first steps of differentiation by regulating the expression of this early gene. On the other hand, at day 4 (Fig. 5, lower panel) only DEGDB still enhanced *Cebpβ* mRNA expression. The expression of *Rxrα* was selectively modified only by Rosiglitazone administration both during the early

(day 2; Fig. 5, upper panel) and mid phase of differentiation (day 4; Fig. 5, lower panel). The expression of *Pparγ2,* the adipogenesis master gene, was markedly increased at day 2 (Fig. 5, upper panel) by DiDP and DEGDB, while Rosiglitazone and TMCP did not exert any effect. At day 4 (Fig. 5, lower panel) all the analysed plasticizers were able to increase *Pparγ2* mRNA expression. Overall, the plasticizer-induced regulation of *Pparγ2* expression on day 2 and 4 was quite similar to the one exerted by Rosiglitazone.

As expected, in the late phase of differentiation (day 8) (Fig. 6), the levels of the *Fabp4* transcript were highly increased by Rosiglitazone. The plasticizers TMCP and DEGDB had also a positive effect (4 and 3.5 folds respectively compared to untreated cells) on the expression of this transcript. *Lpl*, another adipogenesis marker gene belonging to the late phase, was modulated by Rosiglitazone and TMCP at comparable levels (about 8 and 6 folds respectively). Conversely, the expression of both *Fabp4* and *Lpl* was not modified by exposure to the phthalate DiDP.

4. Discussion

Plasticizers and their metabolites are a frequent finding in human biomonitoring data of industrialized countries [41–46]. Published datasets in national surveys referring to the last decade track the coexistence of both dismissed compounds, still present in relevant amounts, and new plasticizers and their metabolites [45,46] that are slowly substituting the former ones. Some of the new plasticizers could represent an emerging class of contaminants, therefore evaluation of their potential biological effects is needed [47].

The results of the present study suggest that plasticizers considered safer alternatives to SVHC may actually affect metabolic processes, such as adipogenesis. We demonstrate

that low nanomolar concentrations of four plasticizers currently used in FCMs manufacturing (namely DiNP, DiDP, DEGDB and TMCP) enhance the ability of 3T3-L1 cells to differentiate into mature adipocytes, as shown by a 1.2-2.3 fold increase in lipid accumulation, depending on the chemical and time window of exposure. Computational analysis shows the capability of these compounds to bind to PPARγ and RXRα, two nuclear receptors specifically involved in the adipogenic transcriptional cascade. Each plasticizer was able to transactivate PPARγ and to modulate the expression of adipogenic marker genes to various extents. By analysing the regulation of *Pparγ2* gene expression exerted by test plasticizers we found a certain similarity to the one exerted by Rosiglitazone, a PPARγ agonist, suggesting some degree of overlapping in the cellular mechanisms involved.

Besides Rosiglitazone, we included also BPA as a useful reference compound in all our experiments, since considerable amount of knowledge has been accumulated from *in vitro* and *in vivo* studies on this plasticizer. While some controversy exists in epidemiological data associating BPA exposure and development of obesity and/or metabolic syndrome in human populations [2,3,48] , several animal studies demonstrate that exposure to BPA can affect adipogenesis [2,3,37]. In addition, a number of studies on 3T3-L1 cells have shown that BPA administered during adipocyte differentiation increases lipid accumulation, generally from 2 to 5 folds compared to control, depending on protocol and dosage [24,49–53]. Our results regarding BPA are in line with most previous literature data.

Phthalate pro-obesogenic effects in the human population are still under investigation. Some studies relate the presence of phthalates in blood samples and urine with an increased risk of obesity and metabolic syndrome [54–57], however in a context of a larger dataset these links seem to have some uncertainty [58]. Differently from epidemiological data, there is

extensive knowledge that phthalates exposure, particularly DEHP and its metabolite mono (2-ethylhexyl) phthalate (MEHP), have negative outcomes on glucose and lipid homeostasis in cellular and animal models [47,58,59]. However, there are scarce or no studies on the emerging phthalate substitutes DiNP and DiDP. We show that low nanomolar concentrations of DiNP and DiDP are able to enhance lipid accumulation from 20% to 80%, depending on the time-frame of administration. While this is the first report showing that DiDP can increase lipidogenesis in 3T3-L1 adipocytes, a previous study reported small statistically significant effects of DiNP on lipid accumulation [24]. Human biomonitoring studies employing metabolites of DiNP and DiDP as biomarkers of exposure, reported median values of 5.10 μg/L (16 nM) for MCiOP (mono carboxy-isooctyl phthalate, a DiNP metabolite) and 2.7 μg/L (7.9 nM) for MCiNP (mono-carboxy-isononyl phthalate, a DiDP metabolite) in the urine of the United States general population (>6 years, 2005-2006 survey, Calafat et al. [60]). These levels found in urine are comparable to the 10 nM concentration used in our experiments. Additional studies compared the levels of phthalate metabolites in urine among mother-child pairs [61,62] showing that children's DiNP and DiDP metabolite excretion was higher than that of the mothers, indicating a possible higher children exposure. In addition, multiple studies [62,63] found a significant temporal decline over the last 15-20 years in urinary levels of metabolites of strictly regulated phthalates (such as DHEP), paralleled by a marked increase in urinary metabolite concentrations of DiNP and DiDP. Given the existing biomonitoring data and the results of our study, further research on the adverse health effects of DiNP and DiDP, including obesity and metabolic dysfunctions, is warranted.

DEGDB is defined by many as a “green plasticizer”. There are currently no published human biomonitoring studies on this chemical and there is only some preliminary evidence of the potential impact of DEGDB on tissue-specific regulation of genes involved in lipid metabolism and energy balance *in vivo* [18]. In our *in vitro* experiments, DEGDB actually showed a lipidogenic effect similar to that of the phthalates DiNP and DiDP. To the best of our knowledge, this is the first report linking this compound to *in vitro*-induced adipogenesis. TMCP belongs to the class of organophosphates, a group of compounds that range from slightly to highly toxic depending on chemical structure, dose and route of exposure [64,65]. Epidemiological data on organophosphates indicate that a prenatal exposure may lead to adverse effects on glucose metabolism at birth [66], but little is known about the outcomes of long-term exposure and adult datasets often report controversial results [67]. Concerning animal studies, recent data show that a chronic or subchronic dietary or perinatal exposure to organophosphates alters metabolic functions causing an obese-like phenotype and a diabetic profile in mice [68–70] and rat models [71,72]. Although there are no data available on TMCP effects in 3T3-L1 adipocytes, recent results on the organophosphate triphenyl phosphate (TPhP) indicate that this molecule is able to increase 3T3-L1 preadipocyte proliferation and subsequent adipocyte differentiation, as well as glucose uptake and lipolysis [73]. TMCP was the most effective compound in enhancing lipid accumulation among the plasticizers we tested. This evidence, together with the fact that this organophosphate showed the highest computational binding affinity and capability to transactivate PPARγ, potentially make TMCP the most obesogenic of the four plasticizers that we tested. Clearly, *in vivo* studies are needed to confirm the plasticizer obesogenic potentials defined *in vitro* in the present study.

To elucidate the mechanism by which DiNP, DiDP, DEGDB and TMCP enhance lipid accumulation in 3T3-L1 cells, we evaluated by computational analysis their interaction with PPARγ and RXRα. *In silico* binding affinity of plasticizers for PPARγ and RXRα receptors was highly indicative of *in vivo* interactions, particularly for TMCP whose K_d values were similar to those calculated for Rosiglitazone. BPA showed a higher K_d value for PPARγ, suggesting an action mainly through other nuclear receptors. The predicted interaction of the plasticizers with PPARγ was confirmed by transient transfection studies and is in line with the significant increase found in the expression of the PPARγ target gene *Fabp4* after exposure to plasticizers during differentiation. These data are in agreement with previous studies suggesting that phthalates and TMCP or TPhP can modulate the regulatory mechanism of lipid metabolism pathways through PPARs and RXRs [15–17,74]. Another significant result from our data is that both DiNP and TMCP show high binding affinity for RXRα. This finding, which is a common trend for other potential obesogens [16,59,75], suggests that binding of these plasticizers to RXRα may independently increase PPARγ transcriptional activity. This possibility, although needing experimental confirmation, is in line with the "permissive" features of the PPARγ/RXRα heterodimer [76], meaning that also RXR ligands can activate it, amplifying the effects on downstream genes. Taken together, *in silico* predictions and transactivation experiments suggest that the mechanisms through which DiNP, DiDP, DEGDB and TMCP increase lipid accumulation involve the direct activation of the PPARγ/RXRα complex.

It is expected that the effect of plasticizers on lipid accumulation in 3T3-L1 cells is linked to and, perhaps, promoted by modifications in the pro-adipogenic transcription factor cascade. In order to test this hypothesis, we analysed a set of transcripts that play a key role in the

adipogenic process: *Cebpβ*, *Rxrα*, *Pparγ2*, *Fabp4* and *Lpl*. In the early phase of adipocyte differentiation, all plasticizers were able to increase *Cebpβ* expression, a transcription factor playing a crucial role in the induction of 3T3-L1 differentiation and required for the binding to genomic adipogenic hotspots of other adipogenic transcription factors [19,77]. CEPBβ is a direct activator of *Pparγ* transcription, therefore an increase in *Cebpβ* expression is expected to reverberate on *Pparγ* expression [77,78]. In line with this, we found that all plasticizers induced also a significant enhancement in *Pparγ2* transcript levels in the middle-late phase of differentiation. Exposure to plasticizers only in the early phase (days 0-2), corresponding to the enhancement of *Cebpβ* expression, was enough to induce a significant increase in lipid accumulation measured at the end of differentiation (day 10). This result suggests that any molecule able to modify the expression and therefore the activity of CEPBβ can have profound consequences on adipocyte differentiation. We can hypothesize that the plasticizers could increase the expression of *Cebpβ* through the activation of the cAMP response element–binding protein (CREB) and the glucocorticoid receptor (GR), however recently additional transcription factors have been found to regulate *Cebpβ* transcription as a consequence of different adipogenic stimuli [21,79]. We observed no changes in the expression of the *Rxrα* gene, except for a moderate increase exerted by Rosiglitazone. Absence of regulation of *Rxrα* expression was somehow expected, since previous studies showed that the human *Rxrα* gene displays features of a housekeeping gene [80]. Additional studies report that RXRα activity is modulated by extensive posttranslational modifications and proteasomal degradation [76], suggesting that RXRα is mainly regulated at the protein level. Like Rosiglitazone, in the late phase of 3T3-L1 cell differentiation TMCP was able to modulate the expression of the adipogenesis marker genes

Lpl and *Fabp4*. Similarly to our result, a recent study [73] reported that 25 μM of the organophosphate TPhP is able to increase 3T3-L1 differentiation by upregulating the expression of *Cebpβ, Pparγ* and *Lpl* during early and mid-late differentiation, respectively. Activation of PPARγ and increased differentiation of 3T3-L1 cells into adipocytes by phthalates (i.e. MHEP and DHEP) has been previously reported [81–83]. Nonetheless, this effects not always correlated with a modulation in late genes involved in lipidogenesis [84]. Similarly, we also observed that DiDP and DEGDB were both unable to modify *Lpl* transcript levels. It is possible that other late genes, not considered in our study, are regulated by these plasticizers. In addition, both DiDP and DEGDB were able to activate PPARγ in transient transfection studies only at the highest concentration (25 μM), therefore showing a lower capability to interact with PPARγ compared to TMCP.

The plasticizer-mediated enhancement of lipid accumulation in 3T3-L1 cells was present when exposing cells both in the early or in the mid-late phase of adipogenic differentiation. However, plasticizers were more effective when added during mid-late differentiation. We can postulate that when plasticizers are delivered in the early phase, they positively modulate *Cebpβ* transcription, leading to enhanced PPARγ expression and receptor availability in the subsequent steps of the lipidogenic process. On the other hand, if plasticizers are added in the mid-late phase, when PPARγ is highly expressed, they can interact directly with this receptor. As a result, lipid accumulation increases even further compared to the early phase treatment. We observed that cells exposed to BPA did not behave differently in the two phases, possibly because of the low BPA binding affinity for PPARγ. Multiple intracellular pathways involved in the induction of adipogenesis by BPA have been described [85,86], mostly characterized by PPARγ/RXRα independent

mechanisms [37,86,87]. The wide range of 3T3-L1 cells responses observed after plasticizer treatments probably reflects not only the multiple pathways engaged by each type of chemical compound [88], but also the high complexity of the cell processes leading to the differentiation into mature adipocytes [20,89].

5. Conclusions

Our study demonstrates that the plasticizers DiNP, DiDP, DEGDB and TMCP, used as safer alternatives to SVHC chemicals, are able to interfere with the adipogenic process in 3T3-L1 cells at low nanomolar concentrations. Our results suggest that the observed increase in lipid accumulation is at least partly mediated by direct binding to the transcription factors PPARγ and RXRα and through regulation of several genes involved in the adipogenic transcriptional cascade. The effect of single chemicals on lipid accumulation was moderate, however it should be considered that multiple plasticizers often occur in the same FCM, therefore the global effect of singularly active plasticizers could be significantly higher in mixtures. For this reason, future studies should address the metabolic effects of mixtures containing TMCP, DiNP, DiDP and DEGDB. Our findings also suggest that these four plasticizers may not be harmless substitute of currently restricted compounds. Given the growing exposure of humans to these plasticizers, further *in vivo* investigation of their effects is warranted.

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Figure and Table captions

Fig. 1. Scalar concentrations of BPA, DiNP, DiDP, DEGDB and TMCP enhance lipid accumulation in differentiated 3T3-L1 cells.

Upper left panel: schematic representation of the experimental protocol (for details, see Materials and Methods section). The blue line indicates the presence of plasticizers (or Rosiglitazone) in the cell culture medium. MDI: differentiation medium; MM: maintenance medium. Graphs show quantification of lipid accumulation by Oil Red O (ORO) staining, elution and absorbance reading. Three independent experiments (n=3) with 3 biological replicates each were carried out. Variations in lipid accumulation were expressed as fold changes of the absorbance of treated cells relative to the absorbance of control cells (=1) ± SEM; *** $p<0.001$.

Fig. 2. Low nanomolar concentrations of plasticizers are more effective in enhancing lipid accumulation when administered during mid-late differentiation.

Upper left panel: schematic representation of the experimental protocol (for details, see Materials and Methods section). The blue lines indicate the presence of plasticizers (0.01 µM) in the cell culture medium. MDI: differentiation medium; MM: maintenance medium. Graphs show quantification of lipid accumulation by Oil Red O (ORO) staining, elution and absorbance reading. 3T3-L1 preadipocytes were treated with plasticizers alternatively from day 0 to day 2 (early differentiation) or from day 2 to day 10 (mid-late differentiation). Three

independent experiments (n=3) were carried out with 3 biological replicates each. Variations in lipid accumulation were expressed as fold changes of the absorbance of treated cells relative to the absorbance of control cells (=1) ± SEM. * differences versus control; $^{\#}$ differences between early and mid-late differentiation; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$.

Fig. 3. TMCP is predicted to interact with both PPARγ and RXRα ligand binding domains.

3D (left side) and 2D (right side) predicted models of the TMCP/PPARγ (A) and TMCP/RXRα (B) complexes obtained by molecular docking. In the 3D representations, the receptor is shown in cartoon mode, whereas TMCP is shown as stick. Predicted non-polar interactions between TMCP and PPARγ Arg^{288} / RXRα Phe^{313} are reported in the 2D schemes. See the Material & Methods section for methodological details.

Fig. 4. Plasticizers can transactivate PPARγ.

HepG2 cells were transfected with pcDNA3-PPARγ, DR1-Luc, and pCMV-β-galactosidase vectors, then were treated with scalar concentrations of Rosiglitazone or plasticizers as described under Material & Methods. Luciferase activities are reported as fold changes of luminescence of treated cells versus control (=1) ± SEM (n=3). * $p<0.05$.

Fig. 5. All plasticizers modulate the expression of *Cebpβ* and *Pparγ2* in the early and/or mid phase of 3T3-L1 pre-adipocyte differentiation.

Left panels: schematic representations of the experimental protocol. The blue line indicates

the presence of plasticizers (25 µM) or Rosiglitazone (100 nM) in the cell culture medium. MDI: differentiation medium; MM: maintenance medium. mRNA expression was evaluated by qReal-Time PCR at day 2 (upper panel) or at day 4 (lower panel). Data are expressed as fold changes in mRNA expression versus control (=1) ± SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Graphs are representative of three independent experiments.

Fig. 6. Among plasticizers, TMCP shows the highest similarity to Rosiglitazone in modulating the expression of late differentiation genes.

Upper left panel: schematic representation of the experimental protocol. The blue line indicates the presence of plasticizers (25 µM) or Rosiglitazone (100 nM) in the cell culture medium. MDI: differentiation medium; MM: maintenance medium. mRNA expression was evaluated by qReal-Time PCR at day 8. Data are expressed as fold changes in mRNA expression versus control (=1) ± SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Graphs are representative of three independent experiments.

Table 1. Primer sequences used for gene expression analysis

Table 2. Predicted equilibrium dissociation constants ($K_{d,pred}$) between PPARγ, RXRα and a set of plasticizers

Table 1

Primers	Sequences
Cebpβ	Forward 5' – CCTGAGTAATCACTTAAAGATGT – 3'
	Reverse 5' – TTTAATGCTCGAAACGGAAA – 3'
Rxrα	Forward 5' – CGGAACAGCGCTCACAGT – 3'
	Reverse 5' – AGCTCCGTCTTGTCCATCTG – 3'
Pparγ2	Forward 5' – CTGTTATGGGTGAAACTCTG – 3'
	Reverse 5' – ATGGCATCTCTGTGTCAA – 3'
Fabp4	Forward 5' – GAATTCGATGAAATCACCGCA – 3'
	Reverse 5' – CTCTTTATTGTGGTCGACTTTCCA – 3'
Lpl	Forward 5' – GATCCGAGTGAAAGCCGGAG – 3'
	Reverse 5' – TTGTTTGTCCAGTGTCAGCCA – 3'
β-actin	Forward 5' – TCTTTGCAGCTCCTTCGTTG – 3'
	Reverse 5' – ACGATGGAGGGGAATACAGC – 3'

Table 2

	$K_{d,pred}$ (M) vs PPARγ	$K_{d,pred}$ (M) vs RXRα
BPA, Bisphenol A	$1.40 \pm 0.34 \times 10^{-6}$	$8.02 \pm 1.38 \times 10^{-7}$
DiNP, Di-isononyl-phthalate	$1.34 \pm 0.24 \times 10^{-7}$	$6.09 \pm 0.98 \times 10^{-8}$
DiDP, Di-isodecyl-phthalate	$1.39 \pm 0.31 \times 10^{-7}$	$1.72 \pm 0.40 \times 10^{-7}$
DEGDB, Diethylene glycol dibenzoate	$5.55 \pm 1.24 \times 10^{-7}$	$3.74 \pm 0.79 \times 10^{-7}$
TMCP, Tri-m-cresyl phosphate	$4.27 \pm 1.26 \times 10^{-8}$	$2.56 \pm 0.40 \times 10^{-8}$
Rosiglitazone, BRL49653	$4.92 \pm 1.43 \times 10^{-8}$	$3.84 \pm 0.72 \times 10^{-8}$

Fig.1

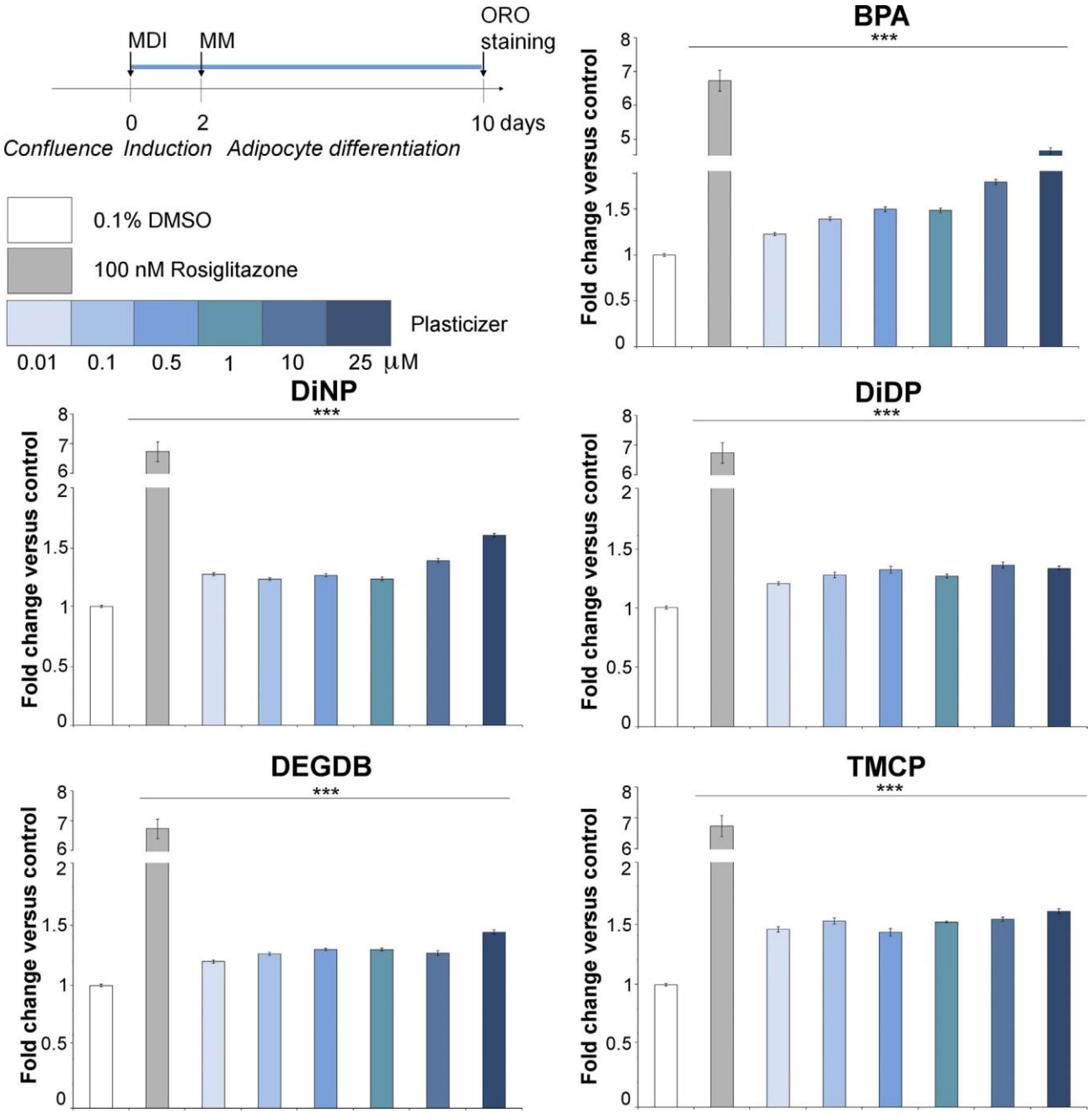

MDI
MM
ORO staining
0
2
10 days
Confluence
Induction
Adipocyte differentiation
0.1% DMSO
100 nM Rosiglitazone
Plasticizer
0.01
0.1
0.5
1
10
25 μM
BPA
DiNP
DiDP
DEGDB
TMCP
***
Fold change versus control

Fig.2

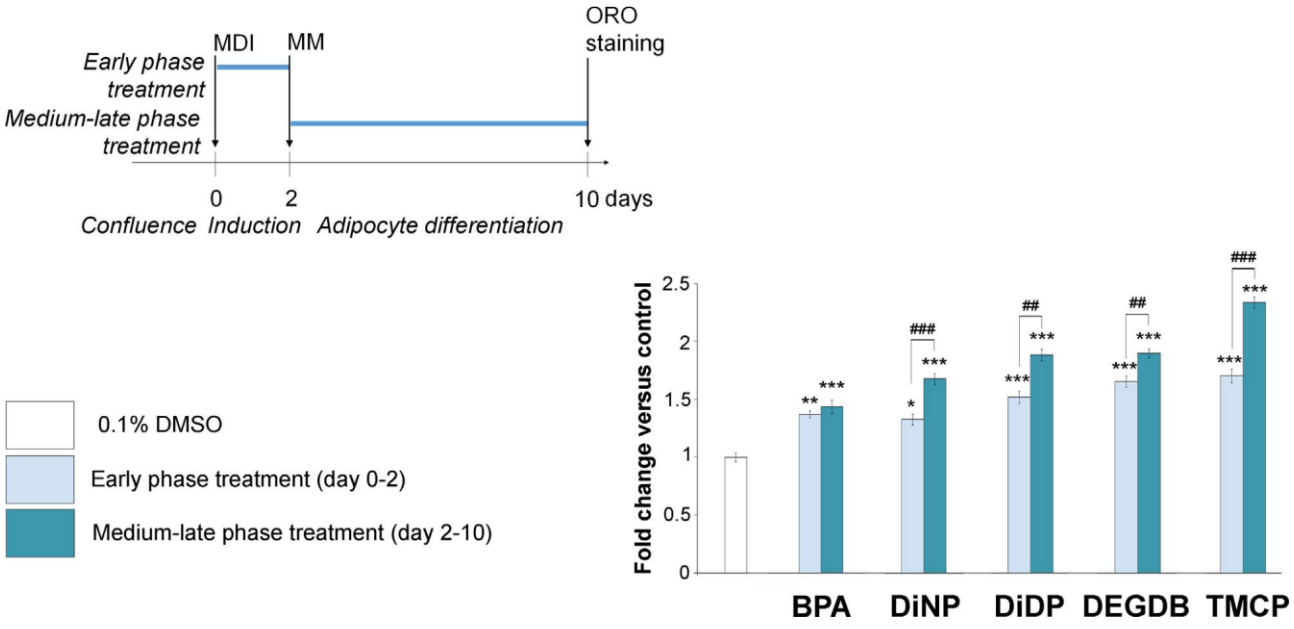

MDI
MM
ORO staining
Early phase treatment
Medium-late phase treatment
0
2
10 days
Confluence
Induction
Adipocyte differentiation
0.1% DMSO
Early phase treatment (day 0-2)
Medium-late phase treatment (day 2-10)
Fold change versus control
2.5
2
1.5
1
0.5
0
BPA
DiNP
DiDP
DEGDB
TMCP

Fig. 3

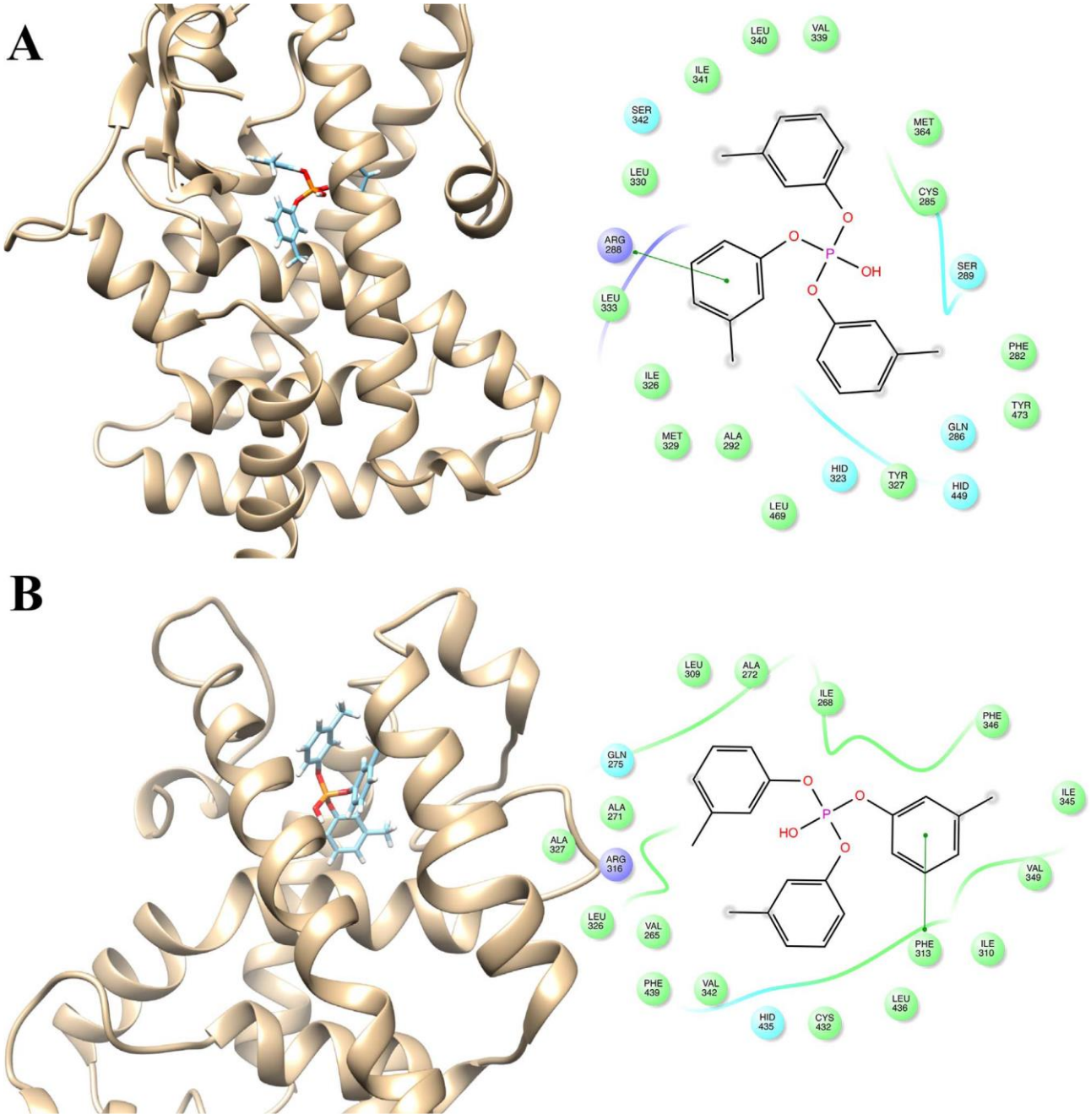
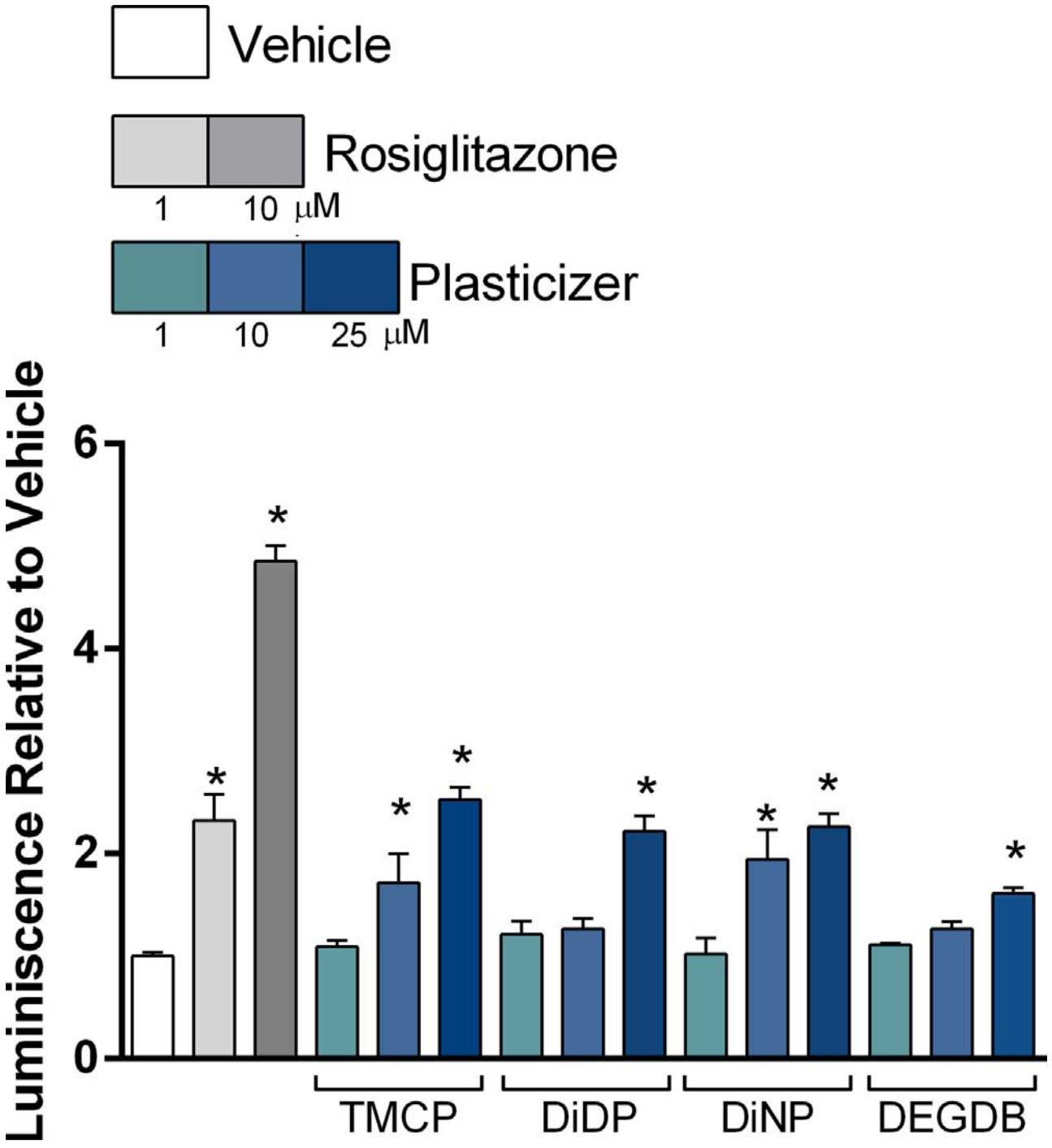
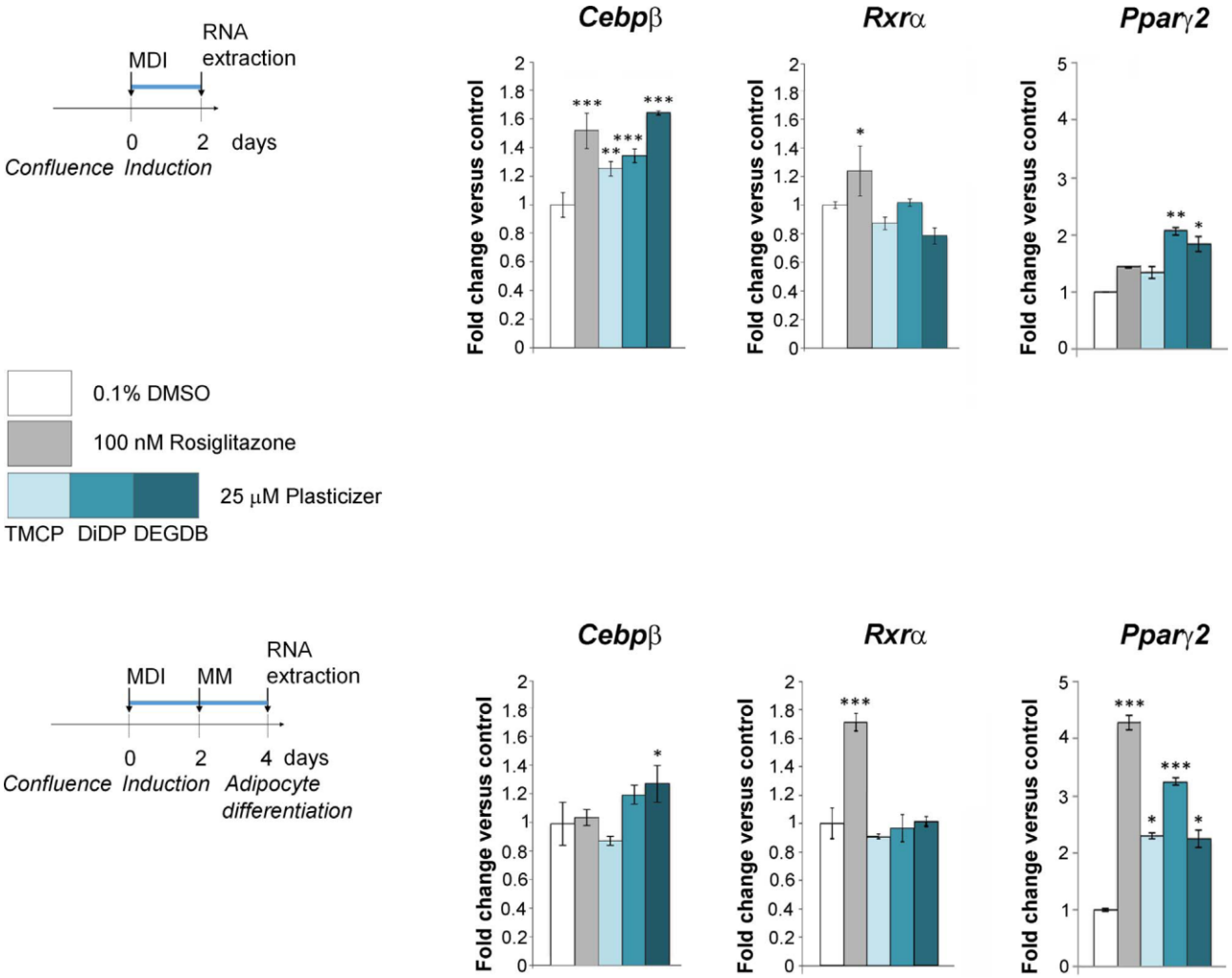

A
LEU 340
VAL 339
ILE 341
SER 342
MET 364
LEU 330
CYS 285
ARG 288
SER 289
LEU 333
PHE 282
ILE 326
TYR 473
GLN 286
MET 329
ALA 292
HID 323
TYR 327
HID 449
LEU 469
B
LEU 309
ALA 272
ILE 268
PHE 346
GLN 275
ALA 271
ILE 345
ALA 327
ARG 316
VAL 349
LEU 326
VAL 265
PHE 313
ILE 310
PHE 439
VAL 342
LEU 436
HID 435
CYS 432

Fig. 4


Vehicle
Rosiglitazone
1
10 μM
Plasticizer
1
10
25 μM
Luminiscence Relative to Vehicle
6
4
2
0
*
TMCP
DiDP
DiNP
DEGDB

1 Fig. 5

2


RNA
extraction
MDI
0
2
days
Confluence
Induction
Cebpβ
Rxrα
Pparγ2
Fold change versus control
0.1% DMSO
100 nM Rosiglitazone
25 μM Plasticizer
TMCP
DiDP
DEGDB
RNA
extraction
MDI
MM
0
2
4 days
Confluence
Induction
Adipocyte
differentiation
Cebpβ
Rxrα
Pparγ2
Fold change versus control

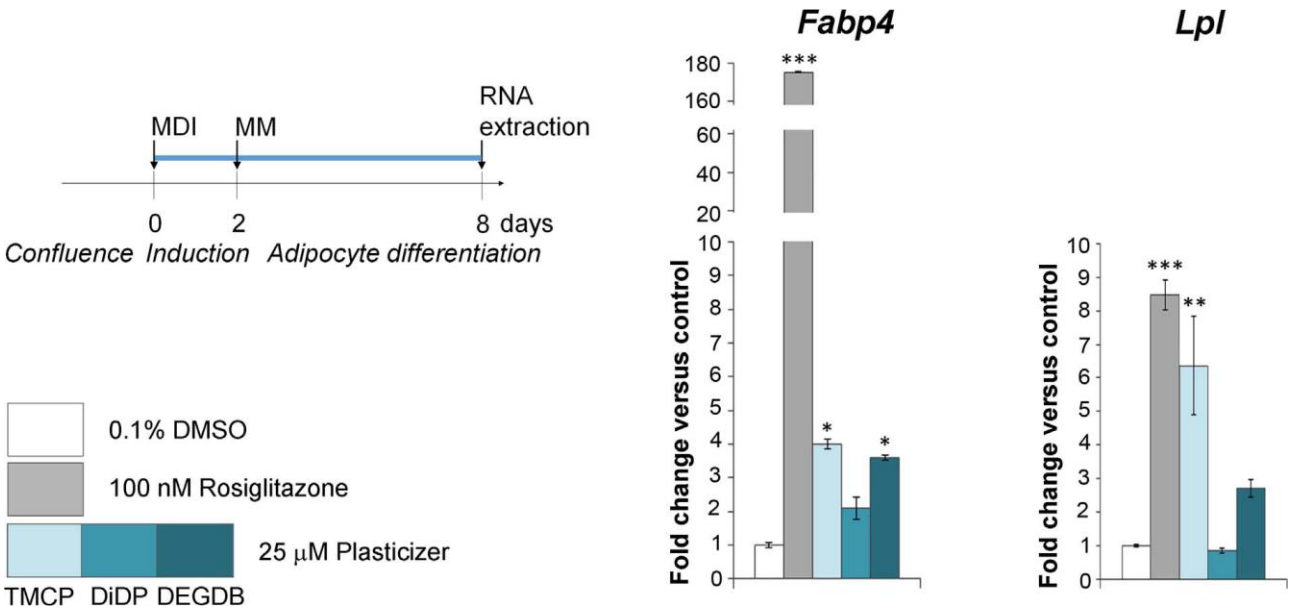
3

4

5

1 Fig. 6

2


MDI
MM
RNA extraction
0
2
8 days
Confluence
Induction
Adipocyte differentiation
0.1% DMSO
100 nM Rosiglitazone
25 μM Plasticizer
TMCP
DiDP
DEGDB
Fabp4
Lpl
Fold change versus control
***
*
**

3